

RESEARCH PAPERS

Interactions of β -Lactoglobulin and α -Lactalbumin with Lipids: A Review¹

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ABSTRACT

Although of excellent nutritional quality, whey proteins are underutilized. To provide new insights for their potential in food products, interactions of α -lactalbumin and β -lactoglobulin with lipids are reviewed. Structural properties of membrane proteins and methods for predicting interactions of proteins with lipids are discussed briefly with regard to lipid-protein complexes in foods. Effects of surfactants on conformations of α -lactalbumin and β -lactoglobulin also are presented. Studies of the interactions of these proteins with synthetic lipids in monolayers and single bilayer vesicles are described. Implications of these studies on the use of whey protein in food emulsions are considered.

INTRODUCTION

Nearly one-third of the milk produced in this country is used to make cheese, resulting in the coproduction of large quantities of whey. Little more than half of the whey is used in the manufacture of human and animal food products, resulting in the loss of more than 45 million kg of whey protein. Protein functionalities such as emulsifying and foaming properties are critically dependent on lipid-protein interactions. A better understanding of these interactions could lead to enhanced utilization of whey and other food proteins. The protein fraction of bovine whey is composed of β -lactoglobulin (β -lg) 50%, α -lactalbumin (α -la) 22%, and a variety of minor proteins. The two major proteins, β -lg and α -la, have been

characterized in great detail and, thus, can serve as model proteins for studies at the molecular level of ways in which proteins interact with lipids in the formation of model membranes, soluble complexes, and micellar complexes.

Membrane proteins are classed as either integral (transmembrane or embedded and projecting from either side of the membrane) or peripheral (adsorbed to the membrane surface or bound to a transmembrane protein). Despite much effort, structural characterization of actual membrane proteins is not complete enough to provide a solid basis for determination of structural-functional relationships (2). Nevertheless, proteins of each type have been studied in enough detail that some generalizations about the types of structures favored by membrane proteins can be made.

For integral membrane proteins, the predominant interactions with the lipid bilayer are nonpolar. Those portions of the polypeptide chain in contact with lipid hydrocarbon chains are organized into regions of regular secondary structure which satisfy the necessity for maximum hydrogen bonding (17). Two types of secondary structure that can serve as transmembrane channels by segregating charged or polar amino acid side chains into the center of the pore while hydrophobic side chains are in contact with the hydrocarbon portion of the lipid (17) are the α -helix and the β -barrel (Figure 1).

The initial interaction between a peripheral membrane protein and the lipid bilayer tends to be electrostatic attraction between charged amino acid residues of the protein and the ionic head groups of the lipid. This electrostatic interaction may be followed by limited insertion of nonpolar amino acid residues between the hydrocarbon tails of the lipid. One proposed structure for the binding of peripheral proteins to membranes is the amphipathic helix (25). According to this model, which also is proposed for lipid binding to plasma lipoproteins, the protein must be able to form an α -helix that has one polar and one nonpolar face. Important

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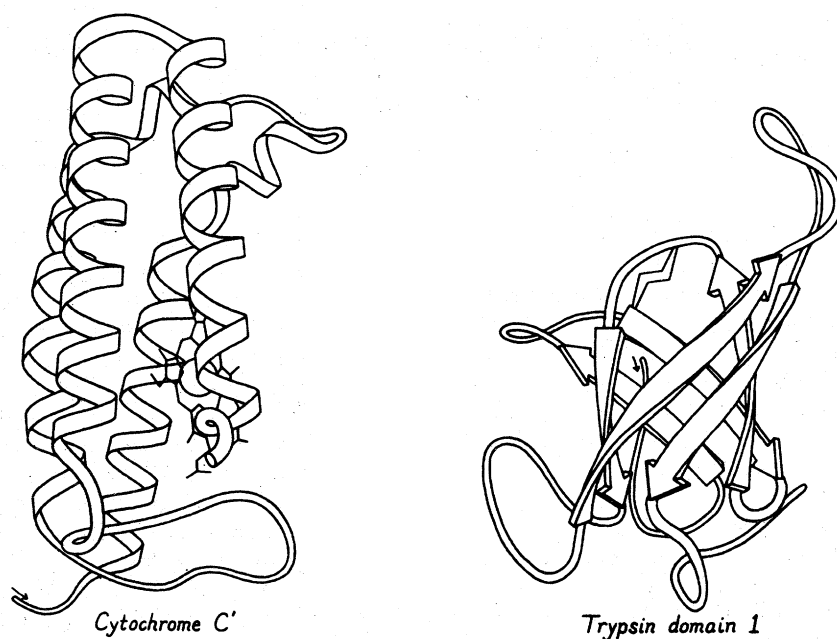


Figure 1. Schematic backbone drawings of α -helices (Cytochrome C') and a β -barrel (Trypsin domain 1) by J. S. Richardson (JSR) are reproduced from (23) with the permission of the copyright holder, JSR.

features of a lipid-binding helix are the presence of at least one ion pair spanning a distance of not more than five amino acid residues in the linear sequence and a high average hydrophobicity for the nonpolar face. This nonpolar face then is interwoven with the hydrocarbon tails of the lipid components while the polar, charged face interacts with the ionic head groups of the lipid.

Predictions

Many proteins that interact with lipids, including the plasma apolipoproteins, have the ability to form such amphipathic helices although they may not be a part of the conformation of the protein in aqueous solution (25). Amino acid residues 6 to 17 of α -la and 130 to 143 of β -lg were identified as potential amphipathic helices with three ion pairs each and average hydrophobicities of about 1400 (3) for the nonpolar faces (25).

The average hydrophobicity of a protein molecule is calculated readily from its amino acid composition (3). However, because many hydrophobic residues are buried in the interior of a native protein, this average may have little

relevance as a predictor of a protein's ability to interact with lipids. Effective or available hydrophobicity based on the behavior of selected proteins in a variety of model systems may be a better predictor (16, 18). Effective hydrophobicities of β -lg, and α -la, and several other proteins are summarized in Table 1, where a loose correlation between these data and the average hydrophobicity of the molecule is seen. Closer correlation is among effective hydrophobicities, where except for lysozyme, results show general trends with little scatter. A specific effective hydrophobicity is, of course, directly related to characteristics of the model lipid probe with which it was determined, so that in using these data one would choose the lipid model most similar to the real system.

Predictions based on amino acid composition, sequence, or correlations with other proteins are a useful starting point, but experimental evidence for interactions of α -la and β -lg with lipids can lead to refinement of predictions for the behavior of whey protein in a food system. A lipid-protein complex that can be isolated free of unbound lipid and protein is the best evidence for binding, but complexes that can be visualized by electron microscopy or that cause

TABLE 1. Hydrophobic characteristics of proteins.

Protein	Hb ^a	log K ^b	R ₄ ^c	R ₆ ^d	S ₀ ^e
β -Lactoglobulin	1070	.39	4.4	12.2	750
α -Lactalbumin	1020	ND ^f	1.7	1.8	ND
Serum albumin (bovine)	1000	.95	10.3	18.2	1400
Ovalbumin	980	.24	1.3	1.5	60
κ -Casein	1110	ND	ND	ND	1300
Lysozyme	890	-.1	7.5	15.1	100

^a Average hydrophobicity calculated from the amino acid composition (3).

^b Effective hydrophobicity determined by hydrophobic partition between an aqueous, pH 7.1, phase and polyethylene-bound palmitate (18).

^c Retention coefficient determined by hydrophobic column chromatography on butylepoxy-sepharose 4B (18).

^d Same as c, but chromatography was on hexylepoxy-sepharose 4B (18).

^e Effective hydrophobicity expressed as the initial slope of the fluorescence increase of *cis*-parinaric acid when titrated with a protein solution (16).

^f ND = Not determined.

changes of physical or spectroscopic properties or in the availability of the components for enzymatic digestion also are accepted as evidence that an interaction has occurred.

Interactions with Surfactants

Mattice (19), in a survey of the effects of sodium dodecyl sulfate (SDS), a surfactant frequently used as a model lipid, on protein conformation, found a small increase of the relative proportion of helical structures in α -la above the critical micelle concentration of SDS at pH 6.9 (Table 2). In the same and other studies, all in the pH 5.5 to 7.0 range (9, 19, 28), the helical content of β -lg was about twice that of the protein in buffer alone. Damon and Kresheck (9) included other surfactants in their study (see Table 2) and established that sodium dodecyl sarcosinate increased the helical content of β -lg nearly as much as did SDS, other ionic detergents increased the helical content to lesser degrees, and nonionic surfactants had little effect on the circular dichroism (CD) spectrum of β -lg. It appears that the initial interaction in this pH range is electrostatic, followed by nonpolar interactions of the protein with the hydrocarbon chain in which longer hydrocarbon chains are more effective than shorter ones.

This preference of β -lg for relatively long hydrocarbon chains was also apparent in studies of free fatty acid binding; Spector and Fletcher (27) found that β -lg had a high affinity for palmitate, which increased as the pH was raised from 6.5 to 8.7 and decreased with increasing ionic strength. Axelsson (1) used partition of protein in aqueous polymeric two-phase systems to study the interaction of β -lg with polymer-bound palmitate. He found points of maximum interaction at pH 4.3 and pH 7.8.

Interactions in Monolayers

Both α -la and β -lg increased the surface area of monolayer films of glyceryl monostearate and glyceryl distearate at pH 6.8, suggesting adsorption of these proteins to the lipids (22). Effects of these two proteins were smaller than those of the caseins, which, lacking disulfide bonds, are less compact. The β -lg does bind stearic acid at this pH, although not as strongly as palmitic acid (27). Borgstrom and Erlanson (4) found that β -lg inhibited the action of porcine pancreatic lipase on tributylglycerol emulsions at pH 7. The mechanism appeared to involve adsorption of β -lg on the lipid surface in competition with the enzyme that binds its substrate by a combination of polar and nonpolar interactions (4).

In monolayer film studies of egg yolk phosphatidic acid (EYPA) and egg yolk phosphatidylcholine (EYPC) with β -lg, Cornell (7) found nonideal behavior for EYPA- β -lg mixtures on subphases at pH 1.3 and 4. The mixed films occupied smaller total areas than were predicted from the pure components. The CD spectrum of a monolayer film of EYPA- β -lg transferred from the pH 4 subphase suggested that β -lg in these films had about 2.5 times as much helical structure as it did in a pure protein film transferred from this same subphase. These results were interpreted primarily in terms of electrostatic attraction between EYPA and β -lg at pH 1.3 and 4. Ideal behavior was obtained for EYPA- β -lg mixtures at pH 6 and for EYPC- β -lg mixtures at any of these pH (7).

Hanssens and Van Cauwelaert (12) found that the nature of the interaction of α -la with phospholipid monolayers was dependent on pH. Increases of the surface pressure of monolayers of phosphatidylserine (PS), dipalmitoyl phosphatidylcholine (DPPC), and cardiolipin occurred when α -la was injected into the subphase, showing that the protein penetrated the monolayer to a greater degree at pH 3 to 4 than at pH 7 to 10. At acid pH, the interaction occurred in two stages, adsorption followed by penetration of hydrophobic amino acid residues

into the monolayer. As pH increased, both penetration at an initial surface pressure and maximum initial surface pressure for penetration decreased. At physiological pH, α -la did not penetrate the monolayer significantly but was adsorbed to the polar head groups and protected them against enzymatic hydrolysis by phospholipase C.

Interactions in Sonicated Systems

α -Lactalbumin with Dimyristoyl Phosphatidylcholine. Sonicated systems frequently are used for studying lipid-protein interactions. Each single bilayer vesicle is like a small closed membrane, and many membrane properties have been studied successfully in such systems. A number of researchers (10, 11, 13, 14) at the Katholieke Universiteit Leuven in Belgium extensively studied interactions of α -la with dimyristoyl phosphatidylcholine (DMPC) vesicles. Hanssens et al. (11) sonicated DMPC in buffered solutions containing .1 M NaCl to form single bilayer vesicles. These vesicles then were mixed with α -la dissolved in the same solvent. There was little change of enthalpy on mixing vesicles with α -la at pH 6 to 7 in a model physiological system. However, at lower ionic strength there was some increase of ΔH . At pH

TABLE 2. Effects of surfactants on the conformation of β -lactoglobulin (β -lg) and α -lactalbumin (α -la).

Protein	Surfactant	S/P ^a	pH	% Helix ^b	Reference
β -lg	None		1-7	10-17	(29)
β -lg	SDS	1600	6.9	35-37	(19)
β -lg	SDS	9000	6.4	34-37	(28)
β -lg	SDS	72	5.5	26	(9)
		144	5.5	38	(9)
β -lg	SDSar ^c	90	5.5	23	(9)
		180	5.5	37	(9)
β -lg	SOctS ^d	1600	5.5	17	(9)
β -lg	DodTAB ^e	270	5.5	24	(9)
α -la	None			24-26	(8)
α -la	SDS	1600	6.9	33	(19)

^aS/P = Molar ratio of surfactant to protein.

^bCalculated from the CD spectrum using the formula: $[\Theta]_{222} = -30300 f_H - 2340$, where $[\Theta]_{222}$ is the molar (mean residue weight) ellipticity at 222 nm, and f_H is the fraction of residues in helical conformations (6).

^cSDSar = Sodium dodecyl sarcosinate.

^dSOctS = Sodium octyl sulfate.

^eDodTAB = Dodecyltrimethylammonium bromide.

4.6, ΔH was independent of ionic strength but increased with increasing lipid to protein molar ratio. The ΔH was temperature dependent in the 15 to 30°C range and in acidic media reached a maximum near the lipid transition temperature (23°C). On gel-filtration chromatography at pH 7, little α -la eluted with the DMPC vesicles, which were identical in size and shape to vesicles in suspensions without protein. However, at pH 4 a DMPC- α -la complex with a molar ratio of lipid to protein of 80:1 was isolated by gel-filtration chromatography. These complexes were slightly smaller than DMPC vesicles and appeared bar shaped rather than spherical in electron micrographs. At neutral pH the intrinsic fluorescence emission of α -la was affected little by DMPC vesicles, suggesting that the tryptophan residues did not penetrate the bilayer enough to change the polarity of their environment. In acidic media, pH 4, the relative fluorescence doubled as the ratio of lipid to protein increased from 0 to 80. At the same time, there was a 4 to 5 nm red shift of the emission maximum. These effects were most pronounced near the lipid transition temperature (23°C). Because α -la is not denatured at pH 4, these observations were interpreted as a swelling of the protein molecule in an apolar environment, reducing the intramolecular fluorescence quenching (11).

Herreman et al. (13, 14) labeled DMPC vesicles by incubating a buffered suspension of vesicles with a solution of 1,6-diphenyl-1,3,5-hexatriene (DPH) in tetrahydrofuran. The incorporation of this probe into the hydrocarbon portion of the vesicles was demonstrated by a large increase of the intensity of fluorescence emission from the DPH. Polarization of the emission from this probe increased when the vesicles were incubated with α -la at 23°C. The increase was greater in acidic solutions than at neutral pH. When the DMPC vesicles were incubated with α -la at 37°C, no increase of fluorescence polarization was observed except in acidic solution (pH 4). The fluorescence polarization measurements were used to determine changes of the lipid transition temperature as a function of both pH and molar ratio of lipid to protein. Transition temperature of DPH labeled DMPC in vesicles increased from 24 to 29°C at pH 4 when the molar ratio DMPC to α -la was 4.25. At pH 7 the increase was negligible (less than 1°C).

Dangreau et al. (10) used a lipid mimicking fluorescence probe, 1,3-di(1-pyrenyl)propane (DPP) with DMPC and α -la to study interactions of lipid and protein. They mixed DPP with DMPC in chloroform, removed the solvent under nitrogen, suspended the film in aqueous buffer, and sonicated to form single bilayer vesicles. Increases of the lipid transition temperature as a function of pH and relative concentration of α -la were comparable to those in (14). Because the tryptophan emission band of α -la overlaps the absorption maximum (348 nm) of DPP, they were able to measure energy transfer between the two species. Energy transfer was observed at pH 4 but not at pH 5, 6, or 7.4. The DPP did not bind to α -la in the absence of DMPC.

Based on these studies, it appears that the kinds of interactions between α -la and DMPC, and probably other lipids, depend on the protein's conformation, which is pH dependent. In sonicated systems at pH 4, stable complexes with a ratio of lipid to protein of about 80:1 are formed. These complexes were smaller than DMPC vesicles, and fluorescence energy transfer measurements suggest significant penetration of the protein into the hydrocarbon portion of the lipid. Perturbation of the phospholipid phase at all temperatures by α -la and insensitivity of complex formation to ionic strength are indications of the hydrophobic nature of the interaction at pH 4 (10). As pH is increased, interaction of α -la with DMPC vesicles follows a two-step mechanism, an initial electrostatic adsorption of the protein onto the lipid surface, followed by a limited conformation change that adds a hydrophobic component to the interaction, except in neutral media where α -la merely adsorbs to the outer surface of the vesicle (10).

β -Lactoglobulin with Dipalmitoyl Phosphatidylcholine. Sonicated mixtures of β -lg with DPPC, DMPC, and EYPC were studied by Brown et al. (5). The behavior of β -lg with each of these lipids, which have hydrocarbon chain lengths comparable to those of milk phospholipids (20), was similar. The DPPC, which forms the most stable vesicles, provided most of the results. No interactions were significant when native β -lg was mixed with phosphatidylcholine vesicles (or multilamellar liposomes) at either pH 3.7 or 7.2 (5). The behavior of mixtures of native β -lg with DPPC vesicles was dependent on pH as is

the behavior of DPPC vesicles without protein present, but it did not appear to involve interactions of lipid with protein. The DPPC vesicle suspensions are much more stable in neutral media than in acid media where vesicle fusion occurs readily.

However, when DPPC and β -lg were combined in a helix-inducing solvent (chloroform:methanol:water:HCl), interactions did occur. The procedure has been described (5). The physical behavior of the DPPC- β -lg complexes after they had been sonicated in the appropriate buffer, containing .14 M KCl, depended on whether the pH of the buffer was above or below the isoionic point (pH 5.2) of the protein. In contrast to results with native β -lg, the solvent-

treated protein seemed to form a protective coating on the DPPC vesicles (200 to 600 Å) formed at pH 3.7 (Figure 2) so that a suspension of these vesicles was much more stable than a similar suspension of DPPC vesicles at this pH. In fact, at pH 3.7 small DPPC vesicles were not stable but fused to form large vesicles (1000 to 1500 Å) (Figure 2). At pH 7.2, inclusion of solvent-treated β -lg with the DPPC led to precipitation of both lipid and protein. From the electron micrographs (Figure 2), it could be seen that vesicles in the 200 to 600 Å size range were formed, but they were flattened rather than spherical, and highly aggregated. From sucrose density gradient centrifugation, with a dilute suspension, a molar ratio of lipid

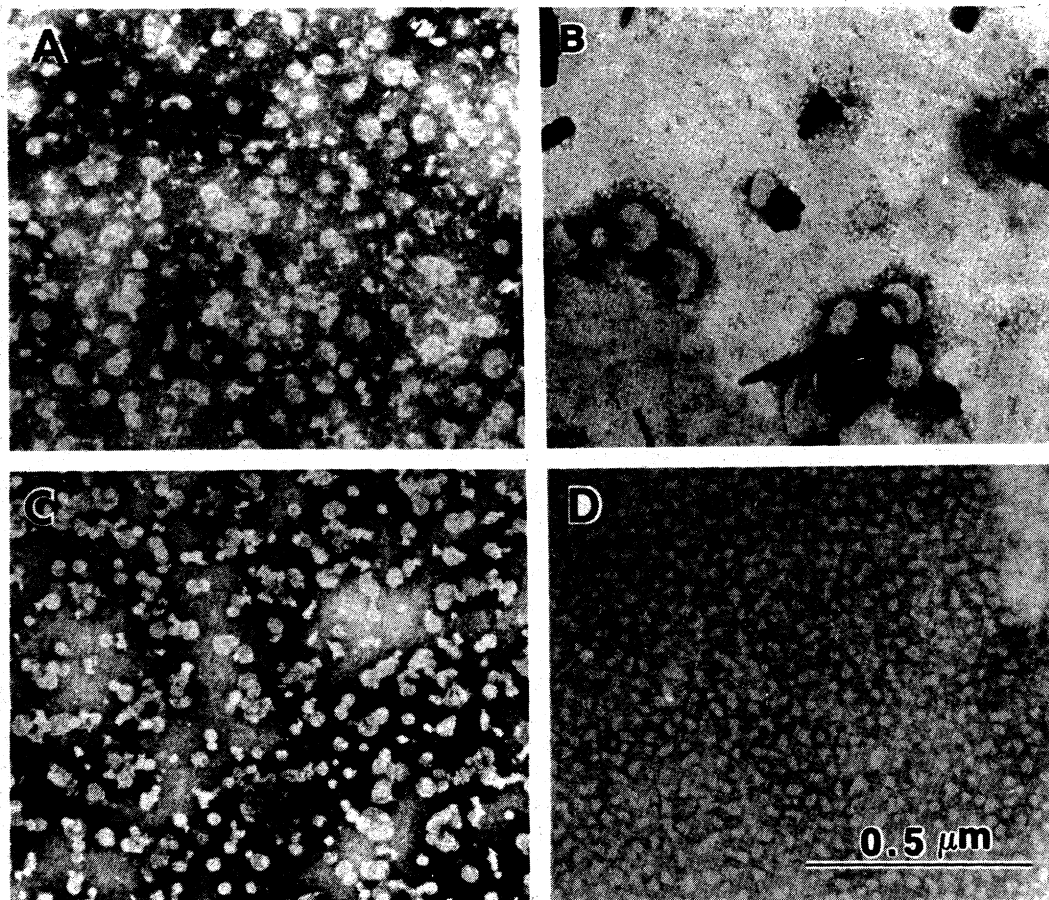


Figure 2. Electron micrographs of phosphotungstic acid-stained vesicles: A) dipalmitoyl phosphatidylcholine (DPPC) with β -lactoglobulin, pH 3.7; B) DPPC, pH 3.7; C) DPPC with β -lactoglobulin, pH 7.2; D) DPPC, pH 7.2. The lipid concentration is .1 mg/ml.

to protein of 20:1 was determined for the sedimenting complex, in agreement with the total SDS binding capacity of β -lg (15).

The far ultraviolet CD spectrum of the DPPC- β -lg vesicles at pH 3.7 suggested that in this complex about 30% of the residues in β -lg are in α -helical structures (Figure 3). Evidence for about 10% α -helix could be found in both the spectrum of solvent-treated β -lg, which had been dried and redissolved in aqueous buffer, and in the spectrum of the native protein. It seems likely that complexation involved formation of one or more segments of amphipathic helix. At pH 7.2, the degree of aggregation was such that no optical spectroscopic measurements could be made.

Further evidence that β -lg protects the DPPC vesicles at pH 3.7 came from the ^{31}P nuclear

magnetic resonance (^{31}P NMR) spectra. Non-sonicated DPPC liposomes at either pH 3.7 or pH 7.2 exhibited an axially symmetric proton decoupled ^{31}P NMR spectrum (Figure 4) characterized by a broad peak with a shoulder approximately 40 ppm downfield, due to the chemical shift anisotropy of phosphorous in an environment with restricted motion (24). This "powder pattern" spectrum typically is obtained with phospholipids in multilamellar liposomes large enough to have sheet-like character. In contrast, sonicated DPPC vesicles, pH 7.2, exhibited only a narrow line 14 ppm downfield from the high-field maximum of the liposome pattern. The ^{31}P NMR spectrum of the DPPC- β -lg vesicles at pH 3.7 closely resembled that of DPPC vesicles at pH 7.2, confirming the presence of small uniform vesicles. At pH 7.2, the ^{31}P NMR spectrum of the DPPC- β -lg vesicles was more complicated. In this spectrum, a vesicle type pattern was superimposed on a much broader band, thus suggesting a greater extent of the interaction of lipid with protein combined with aggregation of vesicles to result in a more restricted environment for the phosphorous atoms. Reappearance of the free vesicle spectrum at higher pH, where the sample became less turbid and electrostatic interactions between DPPC and β -lg became less probable, indicated that fusion had not occurred (5).

In this study β -lg, which had been treated to induce the formation of helical structures, behaved like a peripheral membrane protein at pH 3.7. At this pH, the protein binds electrostatically to the polar head groups of DPPC, and the interaction is stabilized partially by the small additional amount of helix (probably amphipathic) which was in the CD spectrum. At pH 7.2, the interaction between solvent-treated β -lg and DPPC was stronger than at the lower pH. The exact nature of the complex at pH 7.2 was not determined because of the high degree of aggregation of these vesicles.

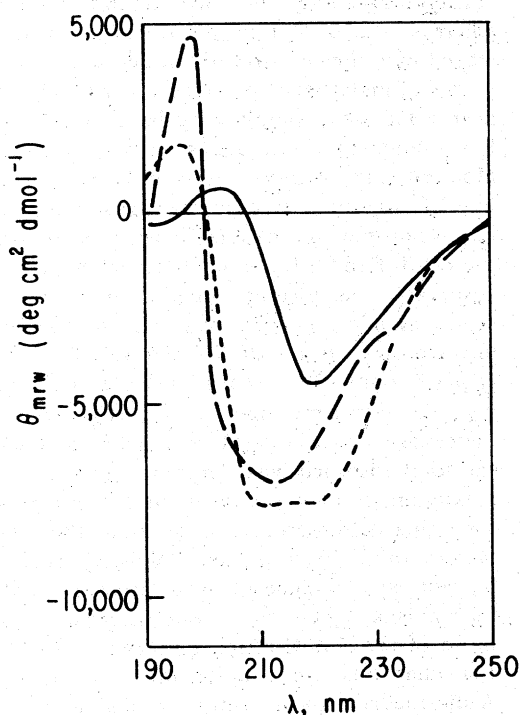


Figure 3. The far-ultraviolet circular dichroism spectra of (—) native β -lactoglobulin (β -lg), (---) denatured, redissolved β -lg, and (- - -) complex of denatured β -lg with dipalmitoyl phosphatidylcholine vesicles. All samples in .02 M acetate, .14 M KCl, pH 3.7 θ_{MRW} is based on a mean residue weight of 113. All spectra shown are averages of at least three separate determinations. A pathlength of .05 cm was used to assure $A < 2.0$ at the λ recorded.

Implications for Food Emulsions

To simplify measurements and their interpretation, most of the model system work on interactions of α -la and β -lg with lipids has used monolayers (4, 7, 12, 22) or single bilayer vesicles (5, 10, 11, 13, 14). The interactions do not, of course, depend on this type of system as can be

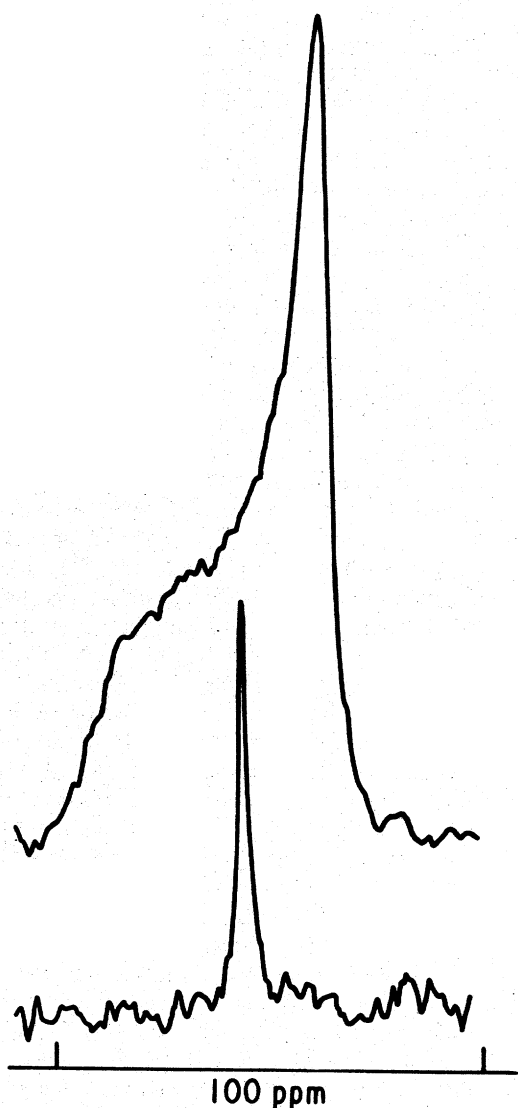


Figure 4. Twenty-four-megahertz proton decoupled ^{31}P NMR (nuclear magnetic resonance) spectra obtained from vortexed liposomes of (upper) dipalmitoyl phosphatidylcholine at pH 7.2; (lower) 1:4 (wt/wt) mixture of β -lg with DPPC at pH 7.2.

seen in the ^{31}P NMR spectrum (Figure 4) of multilamellar liposomes formed by suspending a DPPC- β -lg film in aqueous buffer. The presence of solvent-treated β -lg causes the multilamellar, sheet-like, lipid structure to break into smaller pieces, which tend to curve in on themselves so that the ^{31}P NMR spectrum (Figure 4) collapses into a much narrower line, shifted downfield

from the maximum of the DPPC liposome pattern, similar to the vesicle spectrum.

Native α -la has a greater tendency to complex with zwitterionic phospholipid than does native β -lg in the acid to neutral range common to most food systems. The β -lg of course, may interact with acidic lipids such as EYPA (7) under these conditions. The solvent treatment that alters the conformation of β -lg so that it will interact with neutral phosphatidylcholines seems drastic but is similar to the isolation procedures used to prepare some membrane proteins (21). Possibly a milder treatment such as the use of acidic ethanol would be sufficient.

Yamauchi et al. (30) studied emulsified mixtures of a 6% solution of whey protein and coconut oil at pH 3, 5, 7, and 9. They found that when these emulsions were centrifuged, the amount of protein remaining with the emulsified fat was greatest at pH 5. At pH 7 to 9, the amount of protein in the lipid phase was constant at about one-third of that at pH 5, whereas in acid solution (pH 3) still less protein stayed with the centrifuged fat. Because isoionic points of major whey proteins, α -la and β -lg, are near pH 5, they simply may have been much less soluble in the aqueous phase at this pH. More recently, Shimizu et al. (26) carried this line of inquiry a step further, examining the relative proportions of specific whey proteins in the emulsified fat fraction. Of the whey protein adsorbed to emulsified coconut oil at pH 9, 62% was β -lg and 10% was α -la. As pH was lowered, adsorption of β -lg decreased so that at pH 3, only 13% of the adsorbed protein was β -lg. At the same time, adsorptivity of α -la increased so that at pH 3, α -la made up 48% of the total adsorbed whey protein. There was no maximum in adsorptivity at the isoionic point for either of these proteins. It seems then that factors other than protein solubility are important in adherence of these proteins to the fat emulsion. These results agree in general with those of the DMPC vesicle studies (10, 11, 13, 14), that α -la binds lipids more strongly in acidic media than in neutral or alkaline media, and are consistent with the proposal that α -la behaves more like an integral membrane protein in its lipid-protein interactions at low pH and like a peripheral membrane protein in more alkaline media. Likewise, β -lg, which binds zwitterionic lipids more strongly above pH 7 than below its isoionic point (5) adsorbed

more strongly to the emulsified fat in slightly alkaline media (26).

Both α -lg and β -lg interact with nonpolar, acidic, and zwitterionic lipids under a variety of conditions such as might be useful for food systems. Except for mixtures of native β -lg with neutral lipids, there appears to be some interaction of these proteins with most lipids. The difference in their lipid interaction vs. pH profiles (26) should make their actions complementary and enhance utilization of protein blends or whole whey protein. Because most food manufacturing processes have the effect of at least partially denaturing the protein, use of acceptable processing methods that will tend to encourage helix formation should aid formation of stable lipid-protein emulsions.

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